Gustatory neural processing in the brainstem of the rat

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CHAPTER 5

INTRAMEDULLARY PROJECTIONS OF THE ROSTRAL NUCLEUS OF THE SOLITARY TRACT IN THE RAT: GUSTATORY INFLUENCES ON AUTONOMIC OUT-PUT

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ABSTRACT

The efferent connections of the rostral nucleus of the solitary tract in the rat were studied by anterograde transport of *Phaseolus vulgaris* leucoagglutinin. Rostral to the injection site, fibers travel through the rostral parvocellular reticular formation and deflect medially or laterally around the motor trigeminal nucleus, giving of few terminals in these nuclei and terminate in the parabrachial nucleus. Moderate projections to the peritrigeminal zone, including the intertrigeminal nucleus and the dorsal subcoeruleus nucleus, were observed. Caudally to the injection site, dense innervations from the rostral nucleus of the solitary tract were detected in the parvocellular reticular formation ventral and caudal to the injection site and in the intermediate and ventral medullary reticular formation. The rostral central and ventral subdivision of the NTS up to the level were the nucleus of the solitary tract abuts the fourth ventricle and the hypoglossal nucleus, receive moderate input from the rostral nucleus of the solitary tract. In general, the projections from the rostral nucleus of the solitary tract were bilateral with an ipsilateral predominance. The caudal part of the nucleus of the solitary tract, the dorsal motor nucleus of the vagus and the facial nucleus were not labeled.

It is concluded that medullary rNTS projections participate in oral motor behavior and autonomic control of abdominal organs.

INTRODUCTION

The nucleus of the solitary tract (NTS) is the major integrative visceral sensory relay nucleus in the brainstem. It is the principal recipient of first-order gustatory afferents and visceral afferents from gut, hepatic and pancreatic receptors.

Gustatory information synapses in the rostral NTS (rNTS) and is disseminated by the rNTS along two major pathways. An ascending pathway projects via the parabrachial nucleus (PBN) (in rodents) to several forebrain nuclei, including the thalamus, gustatory neocortex, lateral hypothalamus, amygdala and bed nucleus of the stria terminalis [9,21,31,33]. The second major pathway is locally directed and largely confined to the medulla. These intramedullary connections link oral afferent nerves to the orofacial motor nuclei involved in ingestive behavior and rejection responses [3,6,17,30,35].

Apart from ascending and local projections, the NTS subserves a major visceral efferent function via regulation of autonomic mechanisms [4,12,24,25], including vagal parasympathetic and sympathoadrenal control. Within the NTS, gustatory information may be integrated with incoming visceral afferent responses and thereby influence general autonomic responses. Gustatory stimulation can induce a number of autonomic changes, such as pancreatic insulin release [27,28]. It is obvious that the neuroanatomical architecture of gustatorily induced and vagally mediated responses involves the rNTS and the dorsal motor nucleus of the vagus (DMnX). Furthermore it is confined to the medulla, since decerebration does not significantly alter these mechanisms which persist in decerebrated animals [7,15]. Retrograde transneuronal viral tracing experiments did not substantiate a direct connection between the rNTS and the DMnX and suggested the involvement of intra-NTS connections or the reticular formation [25].

Therefore, in this study efferent projections from the rNTS, within the NTS and within the brainstem were traced with the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (Pha-L). Pha-L was applied iontophoretically in small sites within the rNTS and in the parvocellular reticular formation (PCRt) and the vestibular nucleus (Ve) nucleus which surround the rNTS. Efferent axons from the injection sites were traced and attributed to the rNTS or the nuclei that surround the rostral tip of the NTS.

MATERIAL AND METHODS

Pha-L procedure

The anterograde tracing experiments were carried out on 12 male Wistar rats (of our own breeding) between 3 and 4 months of age. Before injecting Pha-L, the animals were anesthetized with a combination of sodium pentobarbital (i.p., 30 mg/kg body weight), Hypnorm (i.m., 0.5 ml/kg, Janssen Pharmaceutica) and atropine sulphate (i.p., 0.125 mg/kg) and mounted in a stereotactic frame. Pha-L was delivered iontophoretically through beveled glass micropipettes with tip diameters of 15 to 20 µm, which were positioned in the rNTS, the parvocellular reticular formation situated rostrally and ventrally to the rNTS and in the vestibular nuclei situated dorsally to the rNTS. Brain areas were defined by the coordinate system of Paxinos and Watson[19]. The micropipettes were filled with a solution of 2.5% Pha-L (Brunschwig) in 0.01 M phosphate buffered saline (PBS, pH 7.4) and connected to the positive pole of a Midgard CS3 constant current source. Iontophoretic delivery was achieved with a 5-7 µA current for 30 min in a 7 second on and off cycle. After completion of the iontophoresis, the glass pipette was left in its position for 10 min to prevent leakage of tracer in the track during retraction.

Immunocytochemical Staining Procedure

Following a postoperative survival time of seven days the animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg bodyweight) and perfused transcardially with 400 ml of fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4), preceded by a short prerinse with a 0.9% heparinized saline solution. Brains were removed and were cryoprotected by overnight storage at 4 °C in 30% sucrose in 0.1M PB. Transverse or longitudinal sections were cut at 20 µm thickness on a cryostat microtome. The sections were collected in PBS.

Pha-L was visualized with an immunocytochemical avidin-biotin staining technique. Prior to the first antibody incubation, sections were immersed for 10 min in 0.3 % H_2O_2 in 0.01M PBS to exhaust endogenous peroxidase activity. The sections were then incubated for 1 h at room temperature (RT) in 10 % normal goat serum (NGS) to suppress non-specific antibody binding, followed by an incubation for 24 h at 4 °C in a rabbit anti-Pha-L primary antibody solution (1:2000; Brunschwig, 0.5% Triton X-100, 5% NGS). Subsequently, the sections were incubated in 5% NGS for 1 h at RT, in a Goat-anti-Rabbit IgG biotinylated secondary antibody solution (1:200; Zymed, 0.5% TritonX-100, 2.5% NGS) for 90 min at RT. Thereafter, a 90 min. incubation in Streptavidin-HRP (1:200; Zymed) was carried out. Between all steps, the sections were rinsed thoroughly with 0.01M PBS. Finally the sections were rinsed in 0.1 M Tris/HCl (pH 7.6) and processed by the diaminobenzidine (DAB)-H_2O_2 reaction (30 mg DAB and 0.01 % H_2O_2/100 ml 0.1M Tris/HCl, pH 7.6).

After the immunocytochemical staining the sections were mounted, air dried, counterstained with cresyl violet, dehydrated, cleared in xylene and coverslipped with DPX mountant (BDH-Pool, UK).

Analysis

The atlas of the rat brain, according to Paxinos and Watson [19] was used to localize Pha-L labeled neurons, axons and terminals. Tracing results were analyzed by light microscopic examination and visualized by camera lucida drawings.

RESULTS

Cytoarchitecture of the rNTS

The NTS can be divided into three rostro-caudal levels (Fig. 1A). The rostral level extends from the point at which the NTS buds off from the dorsomedial edge of the spinal trigeminal nucleus to the level where the NTS first touches the fourth ventricle. The intermediate NTS extends further caudally to the obex. The caudal NTS extends caudally into the medulla to the junction with the spinal cord. The rNTS is composed of four subdivisions [2,3,8,35]: medial (m), rostral central (rc), rostral lateral (rl) and ventral (v) (Fig. 1B). The mNTS occupies the medial quarter of the rNTS, the v subdivision is positioned ventral to the rc and rl subdivisions of the nucleus.

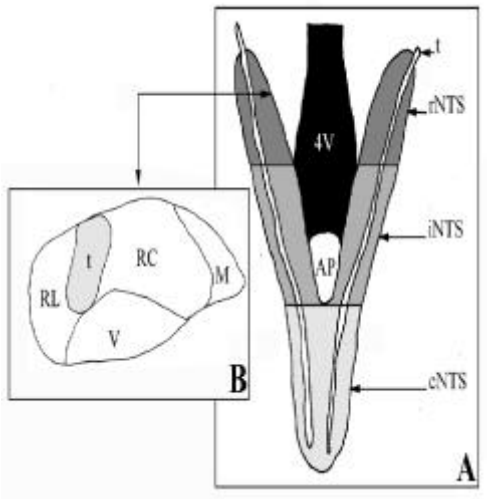


Figure 1. Diagram of the nucleus of the solitary tract (NTS) in horizontal and coronal section, showing the division of the NTS in three rostrocaudal levels (A) and the subnucleuar organization of the rNTS (B). Abbreviations: 4V, fourth ventricle; AP, area postrema; M, medial subnucleus of the rNTS; RC, rostral central subnucleus of the rNTS; RL, rostral lateral subnucleus; (c/i/r)NTS, nucleus of the solitary tract (caudal/intermediate/rostral); t, solitary tract; V, ventral subnucleus of the rNTS.

Injection sites

The NTS was injected at Bregma -11.4, at the level where its lateral subnucleus touches the dorsal tip of the dorsomedial spinal trigeminal nucleus. The effective site of the tracer injection is defined by the presence of neurons filled with tracer. In general the effective injection sites were spherical and their diameter ranged from 400 to 700 µm. Outside these boundaries, filled neurons were occasionally found. Due to the small area of the rNTS at the level of the injection site, several injections comprise both the rNTS and the surrounding area, including the rostral and caudal parvocellular reticular formation (rPCRt and cPCRt) and the vestibular nucleus (Ve), situated rostrally, ventrally and dorsally to the rNTS. Control injections in which the tracer is confined to these surrounding nuclei were made to discern between projections originating within and outside the boundaries of the rNTS. The locations of the injection sites are depicted in Figure 2. Injection areas 1 to 4 represent individual cases (as described below), while the injection areas 5 and 6 are a summary of three cases each.

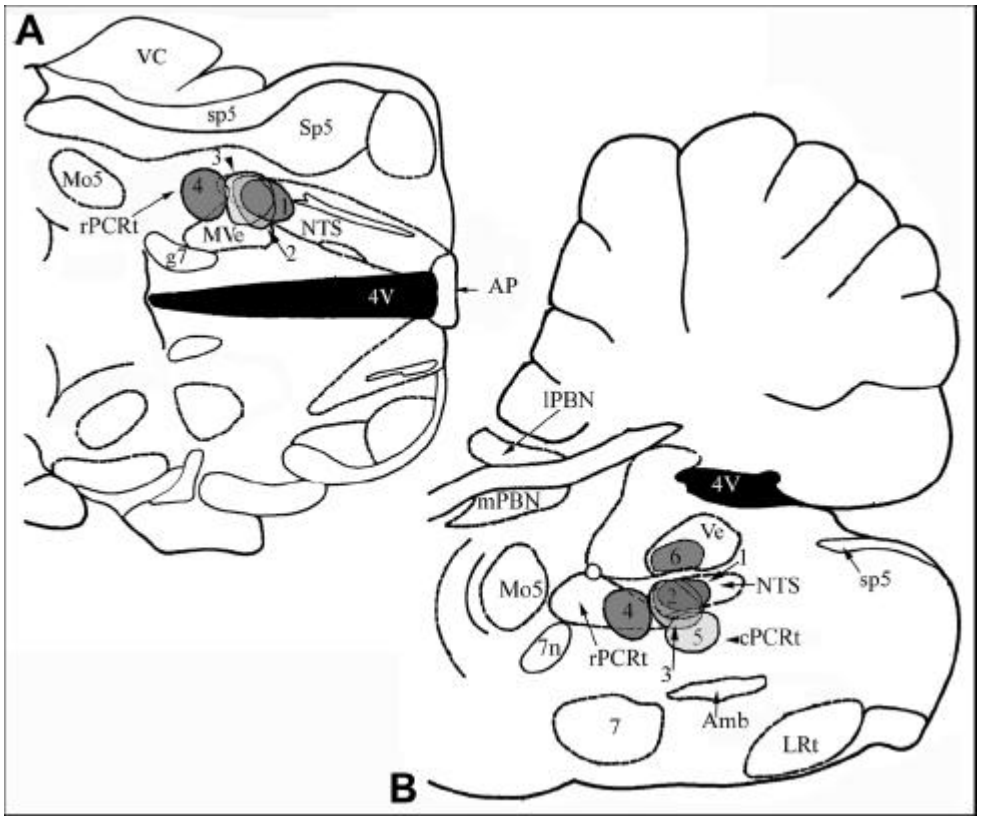


Figure 2. A diagram of a horizontal (A) and a sagittal (B) section, showing the location of the injection sites in and around the rostral nucleus of the solitary tract. Injection areas 1 to 4 represent individual cases, while the injection areas 5 and 6 are a summary of three cases each. Abbreviations: 4V, fourth ventricle; 7, facial nucleus; 7n, facial nerve; Amb, ambiguus nucleus; AP, area postrema; g7, genu facial nerve; Mo5, motor trigeminal nucleus; NTS, nucleus of the solitary tract; (m/l)PBN, parabrachial nucleus (medial/lateral); (c/r)PCRt, parvocellular reticular nucleus (caudal/rostral); sp5, spinal trigeminal nerve; Sp5, nucleus of the spinal trigeminal nerve; VC, ventral cochlear nucleus; Ve, vestibular nucleus.

Pha-L tracing results

An injection site largely confined to the rNTS is exemplified by the case summarized in Figure 3 (case no.1). The injection was largely confined to the rc and v subnuclei of the rNTS (B -11.4). The majority of the labeled axons that emanated from the injection site showed a characteristic rostrolateral and caudal trajectory. The ascending axons traversed in dorsolateral direction through the rPCRt and gave rise to few terminal boutons in the rPCRt, the motor trigeminal nucleus (Mo5) and the principal sensory trigeminal nucleus (Pr5). Most fibers deflect medially or laterally around the Mo5 with terminals in the peritrigeminal zone, the intertrigeminal nucleus and the dorsal subcoeruleus nucleus. Although the more rostral levels were not analyzed, it appeared that most of the axons passing around and through the Mo5 ended as swellings in the medial and lateral

subdivisions of the PBN. Before reaching the Mo5, dorsally to the genu facial nerve, part of the ascending axons branched of laterally and crossed the midline, forming swellings in the contralateral Mo5, Pr5 and surrounding areas to a lesser extent as compared to the ipsilateral site. Furthermore, labeled axons and their varicosities were found in the medial longitudinal fasciculus, within the mesencephalic trigeminal tract and in the superior cerebellar peduncle.

Most of the descending axons leave the rNTS ventrally and course in caudal direction through the parvocellular and intermediate (Irt) medullary reticular formation, giving rise to numerous terminal boutons. The heaviest projection to the PCRt was directly ventral to the injection site (rPCRt) and diminished in ventral direction (cPCRt). At the level of the hypoglossal nucleus (12n), axons form swellings in the dorsal medullary reticular formation (MdV) lateral to 12n. In the caudal medulla the axons coursed to the spinal cord, without the presence of terminal boutons. This labeling pattern was bilateral with an ipsilateral predominance.

A relatively small part of the descending rNTS projections travels through the NTS, mainly in the horizontal plane of the injection site. Pha-L labeling was detectable down to the level where the NTS abuts the fourth ventricle. Within the NTS the sparse descending axons and swellings were mainly located along the medial border of the NTS in the medial and rostral subnuclei of the medial subdivision. Occasionally some anterogradely labeled fibers were found in the tractus. The labeling at the contralateral NTS showed the same pattern and was less numerous as compared to the ipsilateral side.

Some rNTS injections comprised part of the ventrally located PCRt (for about 30% in case no. 2 (Fig. 4) and 50 % in case no. 3 (Fig. 5)). The ascending and descending projection patterns (case no.3, not shown) are very similar to those that can be observed in case no. 1 (Fig. 3), where the axons solely arise from the rNTS. However, additional labeling originating from anterograde transport of Pha-L by PCRt neurons is present. First, a higher amount of labeled axons and swellings were found in the hypoglossal nucleus, especially at its most caudal level in the ventral part, in close vicinity to the DMnX. Furthermore, more terminal labeling was found in the Mo5 and axons and their varicosities were observed to enter the 7n. A considerably higher amount of labeled axons and swellings were detected in the medullary reticular formation (MRF) (including cPCRt, Irt and MdV).

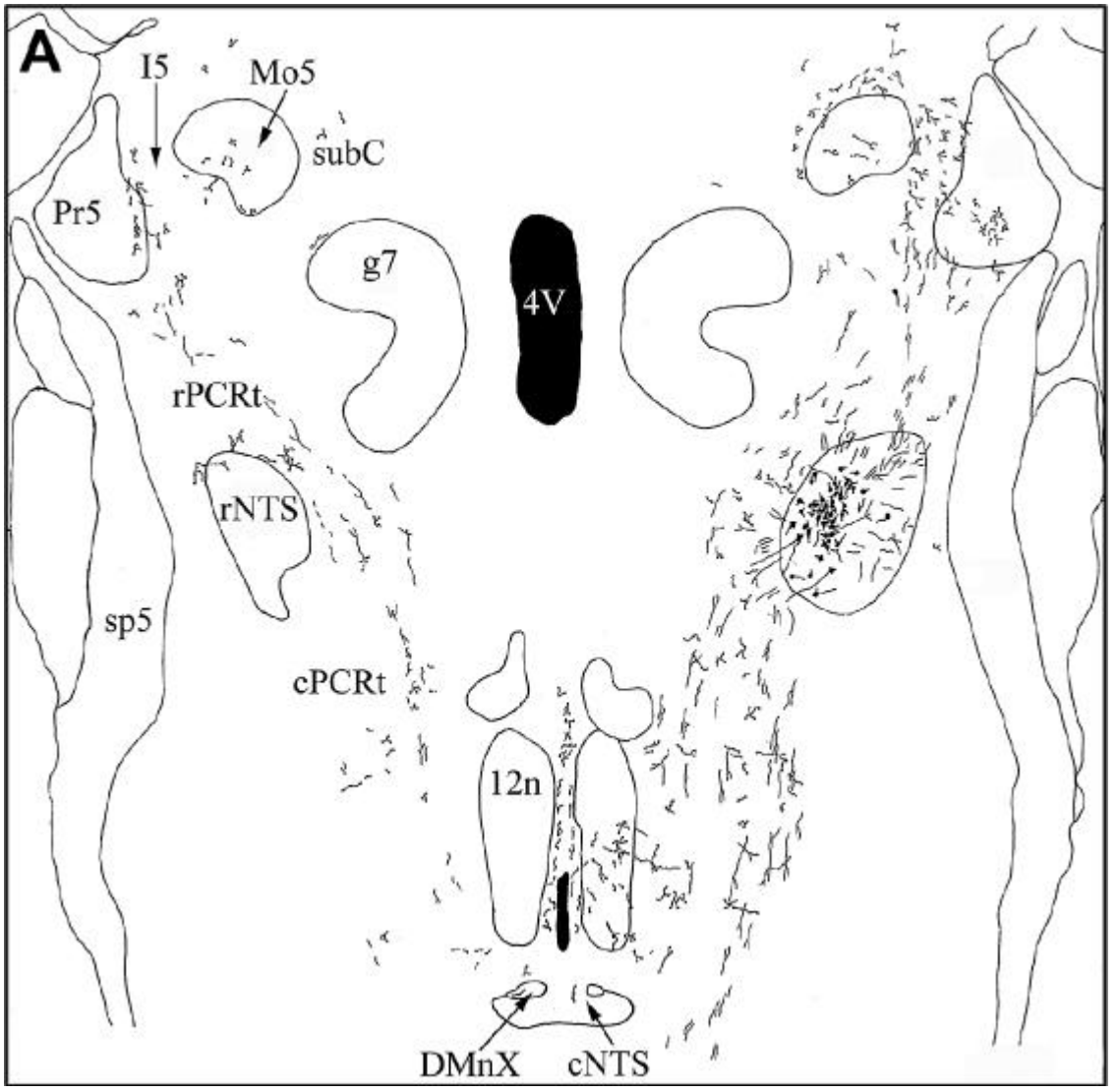


Figure 3. A series (***A***, B, C) of horizontal drawings showing the labeled projections within the brainstem after Pha-L injection in the rostral nucleus of the solitary tract. Abbreviations: see list

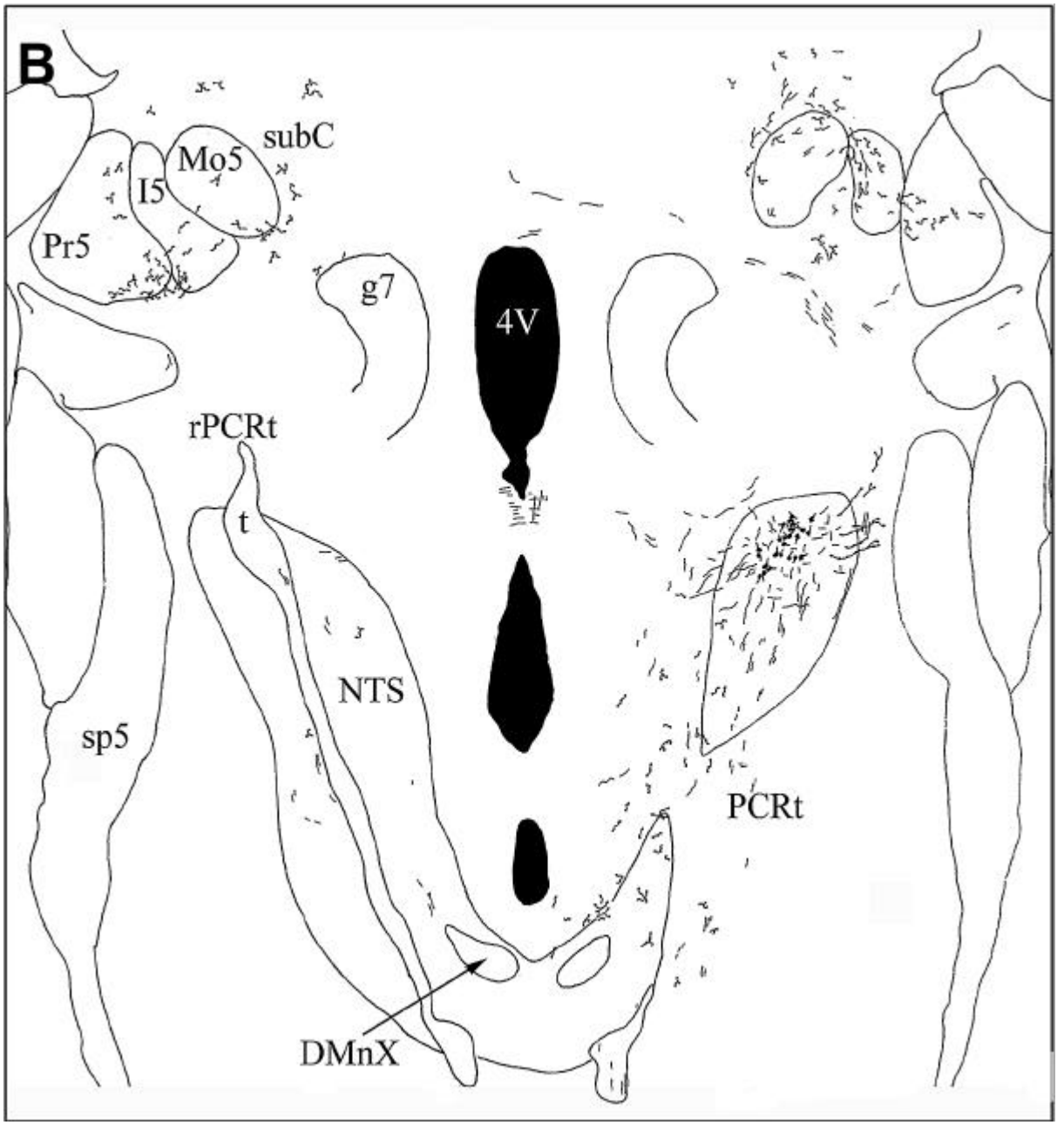


Figure 3. A series (A, ***B***, C) of horizontal drawings showing the labeled projections within the brainstem after Pha-L injection in the rostral nucleus of the solitary tract. Abbreviations: see list.

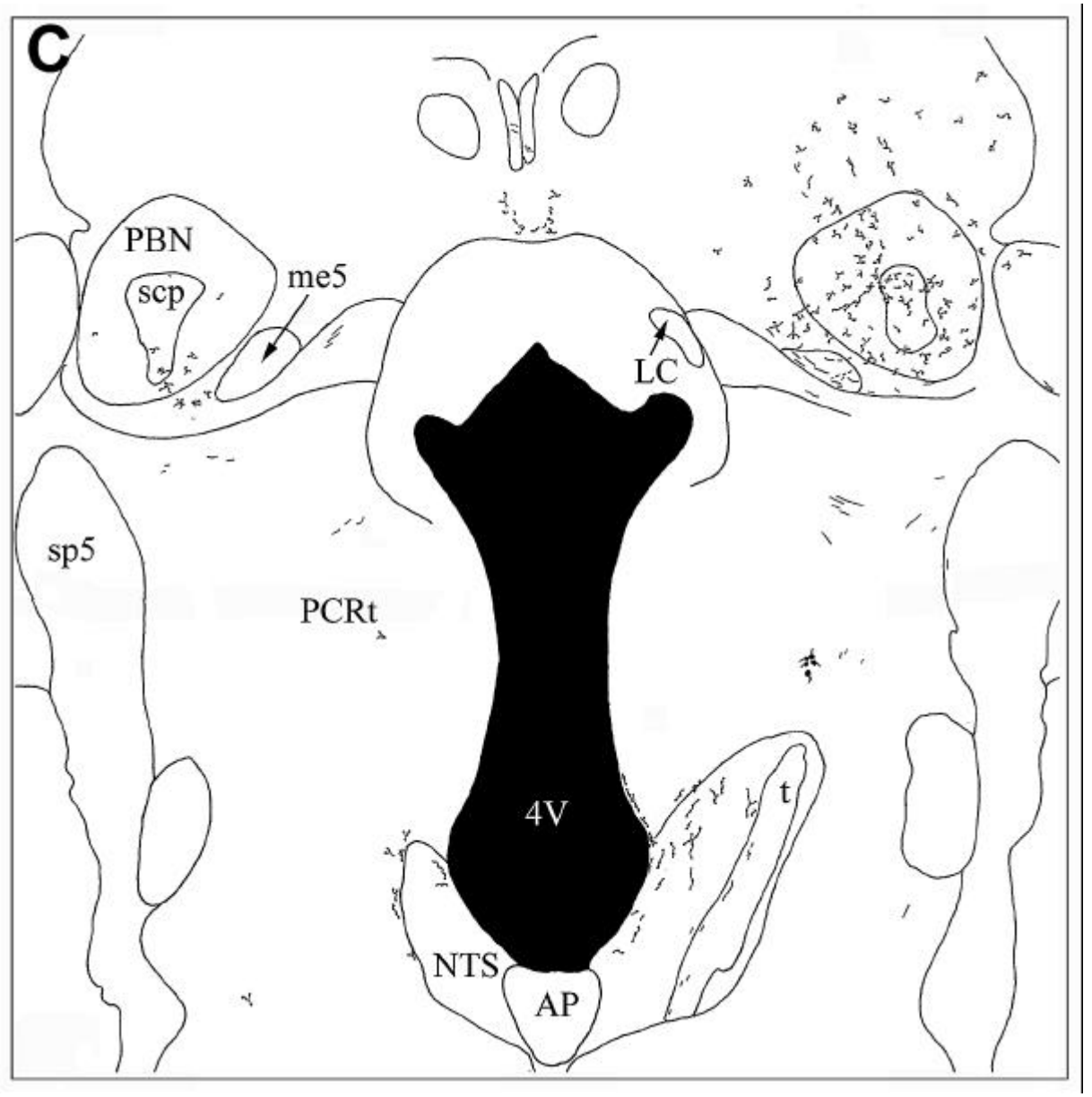
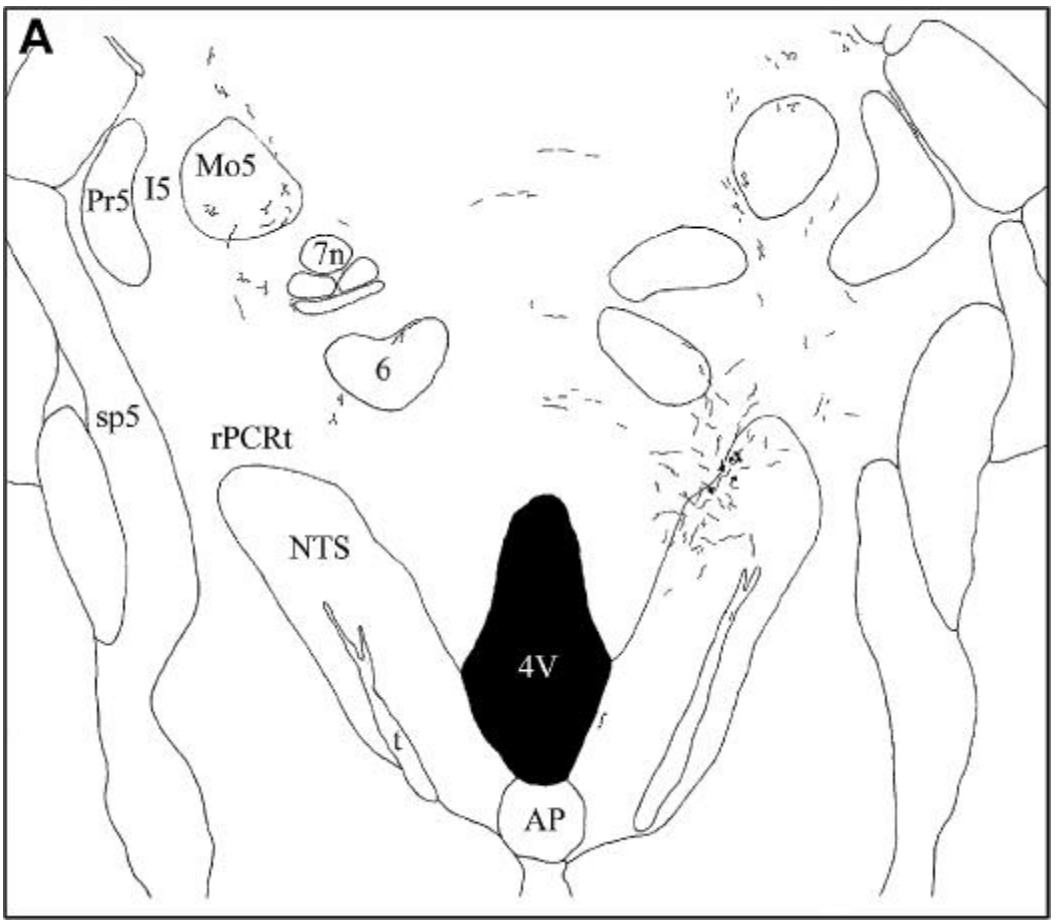


Figure 3. A series (A, B, ***C***) of horizontal drawings showing the labeled projections within the brainstem after Pha-L injection in the rostral nucleus of the solitary tract. Abbreviations: see list.

Figure 4.
A series (***A***, B, C) of horizontal sections which illustrates the efferent connections of the rostral nucleus of the solitary tract and part (30%) of the ventrally located parvocellular reticular formation within the brainstem.
Abbreviations: see list.



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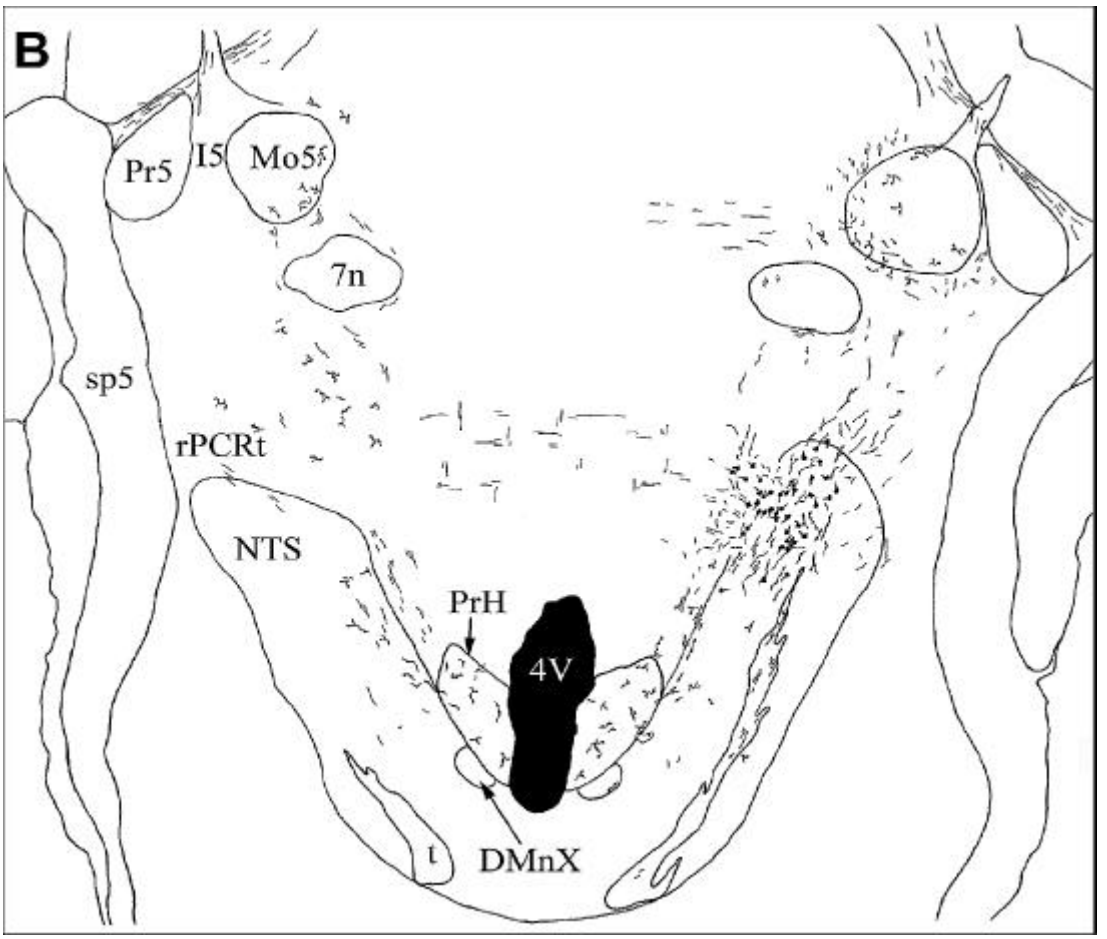
Abbreviations: see list.



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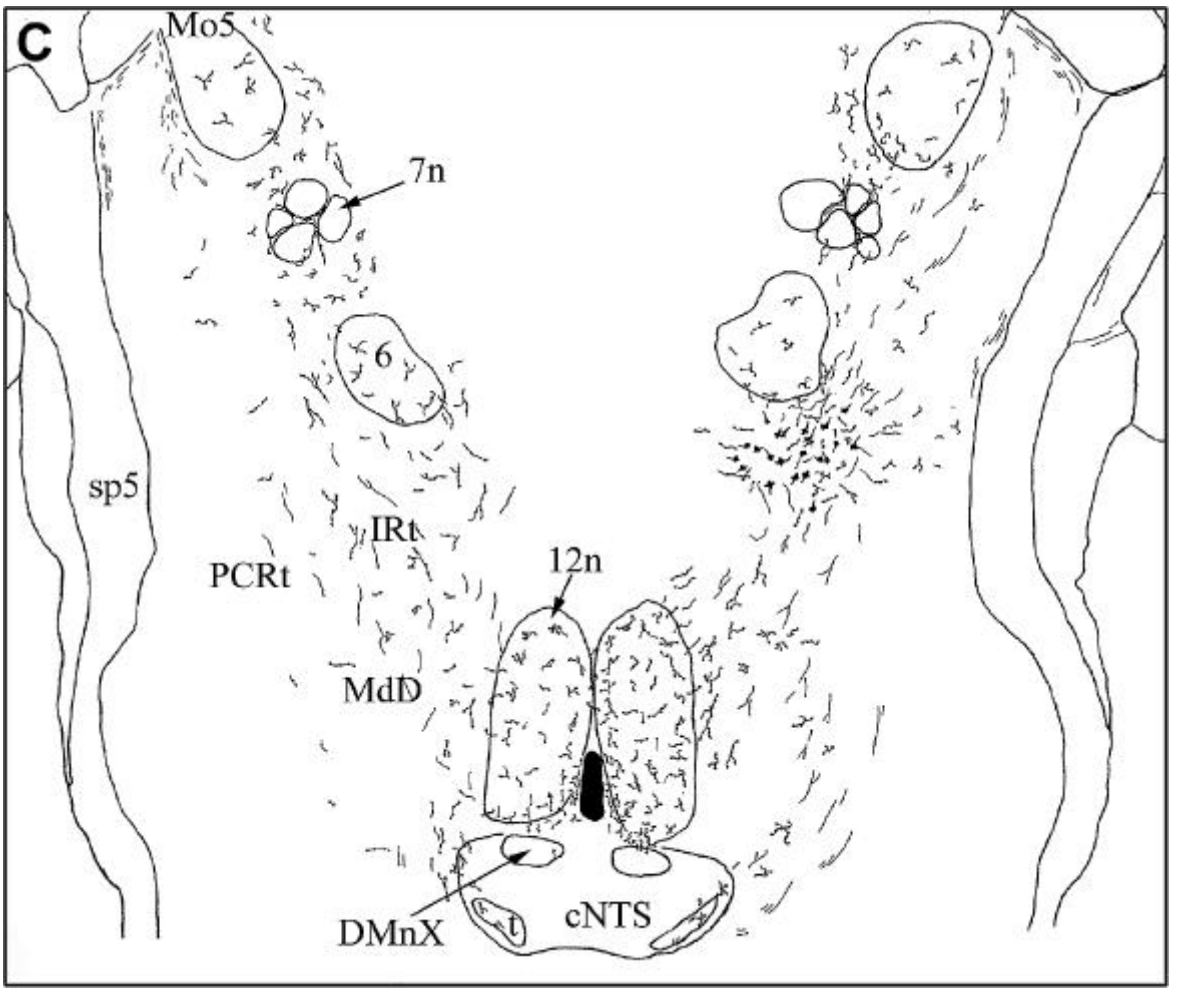
Abbreviations: see list.



Small fibers and terminals were observed in the dorsal DMnX that seemed to emanate from the densely labeled ventral hypoglossal area. DMnX labeling was sparse and confined to its dorsal border. Concerning the intra-NTS labeling, small axons and swellings were detected entering the ventrolateral part of the cNTS from the MdV.

Control Injections

Control injections with Pha-L were positioned in the rPCRt, situated rostrally (n=3), the cPCRt oriented ventrally (n=3) and in the Ve oriented dorsally (n=3) to the rNTS.

Injections confined to the spinal vestibular nucleus, situated dorsally to the rNTS did not reveal projections to the rNTS.

Injections confined to the rPCRt located rostrally to the rNTS (represented by case no. 4, Fig.6) showed extensive projections to the 12n, Mo5 and 7n; the density of the labeling decreased in this order. Projections to the DMnX were present but sparse. Furthermore, axonal projections and terminal boutons were found in the cPCRt and the more caudally located MRF. Projections to the NTS were also observed in the horizontal plane of the injection site. Axons and terminals directly entered the rNTS. At the remaining horizontal levels where the NTS is present (and no injection site was present), the fibers and their varicosities mainly entered the rNTS at its medial and rostral subnuclei and occasionally in and around the tractus. In caudal direction, NTS labeling was still present, but less numerous and more scattered throughout the medial part. The labeling was bilateral with an ipsilateral predominance.

Injections confined to the cPCRt also revealed projections to all oromotor nuclei. The density of the projections to and innervation of these nuclei decreased in the order 12n, Mo5, 7n, nucleus ambiguus (Amb), DMnX. The innervation of the Amb was sparse, but fibers and boutons were clearly visible. Very few fibers were detected at the area postrema (AP) level, in a small strip at the dorsal border between the DMnX and the NTS, from the central canal up to the medial DMnX. A comparable labeling pattern was observed at the contralateral side, although the amount of labeled axons and terminals was lower. cPCRt injections also gave rise to NTS projections, although less compared to the rPCRt. Within the rNTS sparse Pha-L labeled terminals were detected in the medioventral subdivision in the so-called C2 area (adrenergic cells). In caudal direction an occasional fiber with terminals was found in the

medial part of the NTS. At the level of the AP up to the obex, axons and their boutons were found in the ventral part of the lNTS, emanating from the ventrolaterally orientated MdV.

DISCUSSION

In the present study we describe the efferent projections from the rNTS to brainstem nuclei and NTS subnuclei. The rNTS neurons contribute to both an ascending and a descending pathway. Ascending axons travel through the rPCRt, around the Mo5 and terminate in the PBN. The descending pathway is confined to the brainstem. The rNTS efferents appear to terminate mainly in the parvocellular and intermediate parts of the MRF and the 12n and to a lesser extent in more caudally oriented NTS subnuclei.

Anatomical aspects

The results of the present study confirm the main conclusions of previous reports about rNTS efferent projections [3,8,9,17,35]. These studies were the result of anterograde tracing with [^{3}H] leucine and HRP or retrograde tracing with HRP. However, these tracing techniques bear several limitations. A disadvantage of autoradiographic identification of tritiated amino acids is the relatively poor resolution, which rules out discrimination between fibers and presynaptic endings. Furthermore, in both autoradiographic and HRP tracing techniques, tracer always spreads or leaks to nearby regions. In most of the rNTS tracing studies, the injection sites were contaminated with tracer spillage to the surrounding reticular formation.

Since our interest lies in the medullary projections of the rNTS, we will not further discuss ascending projections.

Oromotor nuclei The most substantial medullary projection after Pha-L injection into the rNTS was to the 12n. The other oromotor nuclei received light (Mo5) or no (7n) and (Amb) rNTS input. Other anterograde tracing studies inconsistently report light rNTS projections to the Mo5 and 7n, but usually not to the Amb [2,3,17,35].

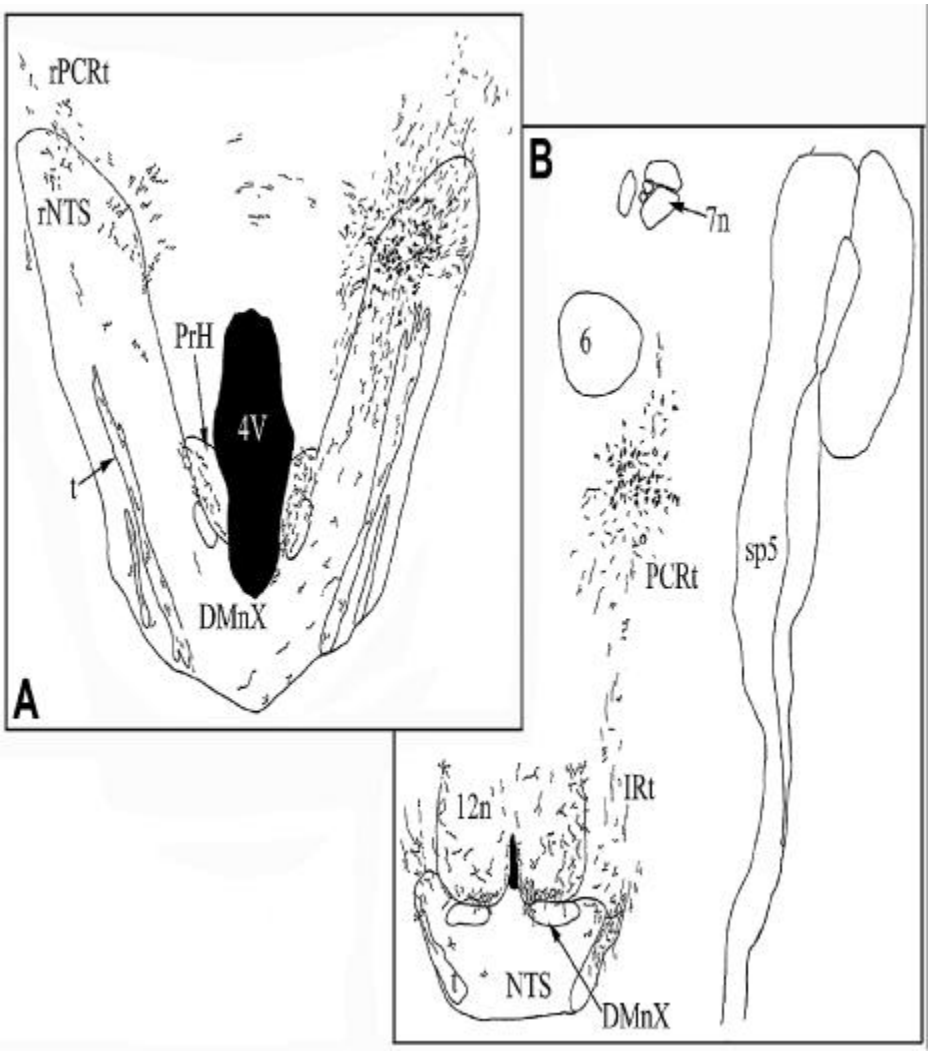


Figure 5. A series (A,B) of horizonta l sections which illustrates the efferen t connections within the NTS after a Pha- L inje ction that covers the rostral nucleus o f the solitary tract (50%) and the ventrall y oriented parvocellular reticular formatio n (50%). Abbreviations: see list.

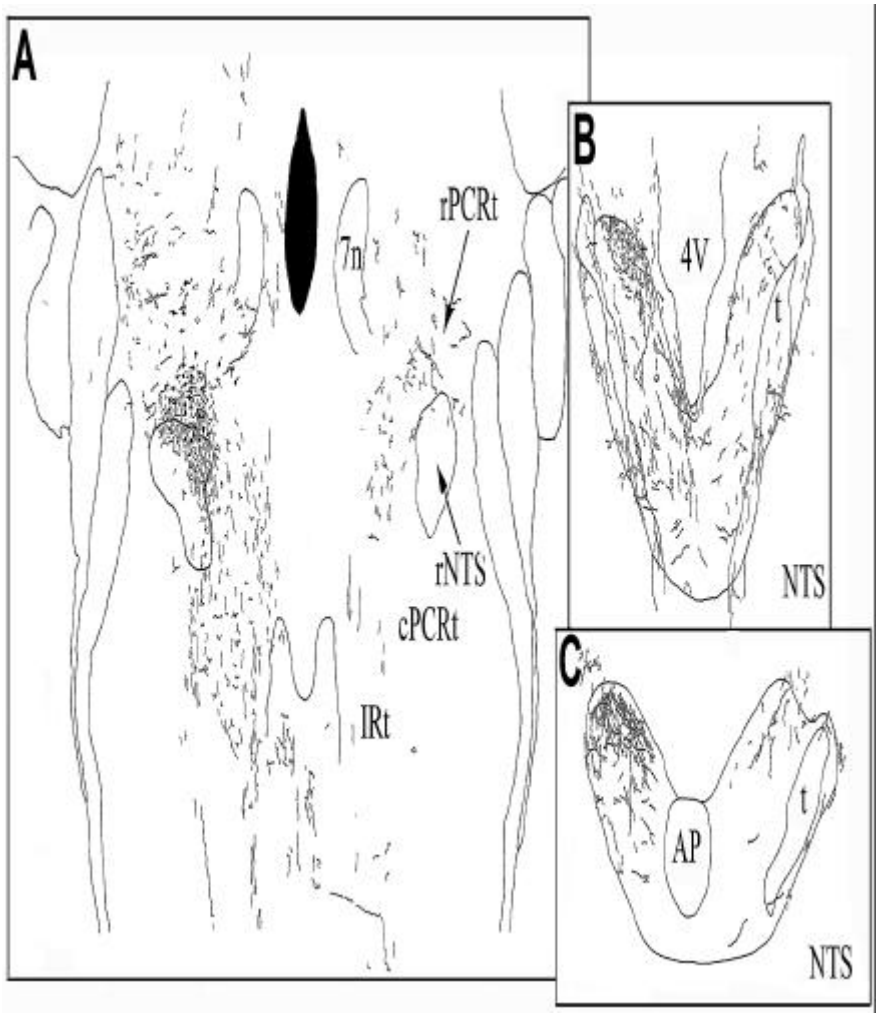


Figure 6. A series of horizontal sections (A,B,C) showing the pattern of labeled efferent connections within the nucleus of the solitary tract, following a Pha-L deposit in the rostral parvocellular reticular formation. Abbreviations: see list.

Additionally, Travers and Norgren [41] failed to show direct projections from the rNTS to the 7n and Amb after retrograde HRP deposits in these oromotor nuclei. When our injection comprises both the rNTS and the ventrally located PCRt (which belongs to the rPCRt), a higher amount of labeled axons and swellings was observed in the Mo5 and 12n. Additional labeling was detected in the 7n, while sparse Pha-L deposits were seen in the DmnX (Fig. 4, 5). cPCRt control injections showed a resembling projection pattern to the oromotor nuclei and also to the Amb. These results partially agree with former tracing studies. Anterograde tracing results have shown that the cPCRt innervates the oromotor nuclei , the Amb and DMnX [3,14,22,32,41]. However, a detailed anterograde Pha-L tracer study [30] showed that caudal and rostral parts of the PCRt project to the Amb, DMnX and intermediolateral cell column (IML) and to the Mo5, 7n and 12n, respectively. Taken together, these results indicate that the rNTS solely projects to the 12n and more lightly to the Mo5. Tracer leakage to the ventrally or rostrally located rPCRt and the cPCRt probably account for the inconsistent results reported so far, concerning the presence and density of 12n, Mo5, 7n, DMnX and Amb labeling.

Medullary reticular formation Projections from the rNTS to the MRF (cPCRt, IRt, MdV) extended over a relatively large area. The heaviest projection was to the PCRt immediately ventral to the injection site. Again, injections that comprised the rPCRt rostral and ventral to the rNTS and the cPCRt showed a higher amount of Pha-L within the MRF. Intra-PCRt projections (rostral to caudal) probably cause this increase in MRF Pha-L labeling [30].

Intra-NTS projections Several studies have shown that within the rNTS, ascending and descending projections originate from different subdivisions [8,35]. The rc division is the major recipient of the chorda tympani and glossopharyngeal nerves and mainly projects to the PBN. The v subdivision of the rNTS projects heavily to the MRF [3,8] and receives afferent input from the rc subdivision. Since our injection sites within the rNTS comprise both the rc and v subdivisions, attribution of efferent projections to either of these subdivisions is not possible. Rostral to caudal NTS projections were found down to the level were the NTS abuts the fourth ventricle, in the horizontal plane of the injection site, mainly along the medial border of the NTS. This finding is in agreement

with some tracing studies [3,35]. Others mention Pha-L labeling in cNTS as well [2,17], which might be the result of spilling of the tracer to the PCRt, since our injection sites that comprise both the rNTS and the surrounding PCRt showed projection to cNTS subdivisions.

Functional considerations

The functional significance of intramedullary projections from the rNTS is still not elucidated. However, it is likely that these projections convey both gustatory and somatosensory information that regulates or influences oral motor behavior. Furthermore, the rNTS is involved in the maintenance of metabolic homeostasis through autonomic control of abdominal organs.

Gustatory stimuli can elicit a number of behavioral and physiological responses to feeding, such as licking, swallowing and active rejection [40]. Behavioral experiments [38] showed that taste nerve section can disrupt the patterns of motor activity associated with licking, gaping and swallowing. This study gives neuroanatomical evidence for taste influence on oral motor behavior through its projections on the oromotor nuclei. Our results show that the hypoglossal motoneurons innervating the tongue musculature [34,36] are directly innervated by the rNTS. The facial and trigeminal motor nuclei, responsible for control of the muscles of face, lips and jaw [34,36], receive indirect rNTS input via rNTS efferents to the MRF. These 'premotor' cells are concentrated in the PCRt ventral to the rNTS (rPCRt) and the reticular formation lateral to the 12n (MdV). The rPCRt projects heavily onto the oromotor nuclei (this study)[30,41]. Furthermore, electrophysiological studies showed that the MdV contains neurons that are active during licking and swallowing [1,11,37,39].

The influence of gustatory information on autonomic responses, such as salivation or the cephalic phase insulin response, always seems to be indirect. The rNTS projections to the MRF may indirectly influence salivation. Preganglionic parasympathetic neurons from the superior salivatory nucleus are found in the rPCRt which is located ventral to and receives input from the rNTS [10,16,42]. Furthermore, the medullary reticular formation (cPCRt, Irt, MdV) that receives input from the rNTS, directly projects to the preganglionic para- and orthosympathetic neurons of the DMnX, Amb and IML [5,14,22,29,32]. The hypothalamus, playing an important role in metabolic homeostasis, projects to the caudally located PCRt. The descending efferents from hypothalamic nuclei that regulate the motivational aspects of food intake mainly innervate the cPCRt [13,14,29,32].

Since our results also show rNTS projections to the iNTS at the level of the

fourth ventricle, the descending gustatory pathway may reach the DMnX through the MRF or through more caudally located NTS subnuclei. This study provides no direct evidence that rNTS neurons projecting to the MRF and the iNTS have a gustatory function. Although the DMnX receives input from both the cPCRt [22,32] and iNTS [5,18,23], there is little evidence for gustatory responses in the MRF and iNTS. In an intracellular recording Renehan and colleagues [20] observed taste responsive neurons in the rNTS that projected to the RF after filling with neurobiotin. Halsell *et al.* [8] report that they often tried to record taste responsive neurons in the RF but found only few extracellularly recorded gustatory responses in the RF. They suggested that the influence of taste may be "modulatory or refractory to observation under anesthesia". This may also be true for taste responsive neurons in the NTS at fourth ventricle level (*i.e.* iNTS), since we were not able to record single-unit responses to whole-mouth gustatory stimulation in this region (unpublished results). In an earlier study, where c-*fos* expression was used to localize neuronal activity caused by gustatory stimulation, no PCRt labeling was found[26]. However, we did observe Fos protein within the iNTS. This could be an indication that descending gustatory information reaches the DMnX through the iNTS.

In conclusion, the anatomical evidence shows that, in the rat, medullary rNTS projections mainly relay in the rPCRt ventral to the rNTS and in the MRF caudal to the rNTS (including the cPCRt, IRt and MdV). A small portion of rNTS efferents appears to synapse in the 12n and in more caudally situated NTS subdivisions. Medullary rNTS projections seem to play a role in oral motor behavior and autonomic control of abdominal organs.

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